

IS THE RECEPTOR-MEDIATED ENDOCYTOSIS OF CHOLERA TOXIN A PRE-REQUISITE FOR ITS ACTIVATION OF ADENYLATE CYCLASE IN INTACT RAT HEPATOCYTES?

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1. Introduction

Cholera toxin exerts its effects on target cells by irreversibly activating the enzyme adenylate cyclase [1,2]. This toxin consists of two non-identical subunits which are held together by non-covalent bonds. The A subunit consists of two non-identical peptide chains, A₁ and A₂, of which A₁ is the active peptide responsible for catalysing the NAD-dependent ribosylation of the guanine nucleotide regulatory unit of adenylate cyclase [3]. Associated with the two A peptides are 5 identical B subunits which each can bind to a G_{m1} ganglioside. These glycolipids act as a cell surface receptor for the toxin molecule.

In isolated membranes either cholera toxin itself or the isolated A subunit can activate adenylate cyclase directly [4,5]. However in intact cells there is a characteristic lag period before the onset of activation of adenylate cyclase [1,2]. The duration of this lag period in intact hepatocytes is dependent upon cholera toxin concentration and upon the fluidity of the cell plasma membrane [6]. The lag time has also been shown to be related to the cell surface redistribution of fluorescent-labelled cholera toxin [7,8]. These observations led to the suggestion [6] that the lateral redistribution of bound toxin may be of importance to the process that allows the A subunit to gain access to the cytosol surface of the plasma membrane where it can act on the guanine nucleotide regulatory unit of adenylate cyclase.

Receptor-mediated endocytosis has been shown to be an important mechanism by which cells rapidly bind and internalise specific extracellular ligands [9–12]. A number of compounds interfere with aspects of this process [11], some of which are the lysomotropic agents, which specifically elevate the

pH in lysosomes [13]. This study investigates the action of a number of such compounds on the cholera toxin-mediated activation of adenylate cyclase in intact hepatocytes.

2. Materials and methods

Adenylate cyclase was assayed by a modification [15] of the procedure in [14].

Isolated hepatocytes were prepared from 24 h starved, 250–300 g male Sprague-Dawley rats [16] and incubated in the medium of [17]. Cells (3–5 mg dry wt/ml) were pre-incubated for 20 min with lactate (10 mM) and in some instances with the test ligands prior to the addition of cholera toxin (10 µg/ml). Incubations, gassing, sampling and the treatment of samples were as in [6].

Cholera toxin, lactate, chloroquine, dansyl cadaverine and methylamine were from Sigma. Enzymes and other biochemicals were from Boehringer. All other chemicals were of AR grade from BDH.

3. Results and discussion

When isolated hepatocytes are exposed to cholera toxin (10 µg/ml) there is a lag period of ~12 min before activation of adenylate cyclase begins (fig.1). The extent of this lag period is believed to be related to the process by which the A subunit of cholera toxin at the extracellular surface gains access to adenylate cyclase at the intracellular surface of the plasma membrane [1,2,18,19]. It has been suggested [18] that the binding of the B subunit to the membrane causes a conformational change in the toxin

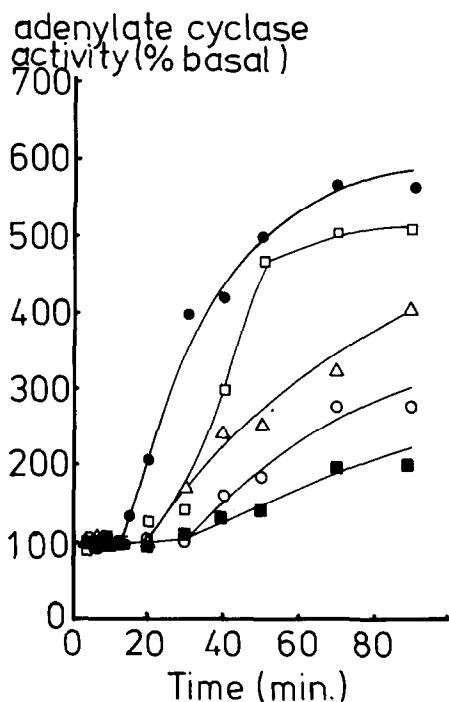


Fig.1. The action of lysosomotropic agents on the activation of adenylate cyclase in intact hepatocytes. Cells were pre-treated as described in the text: control (●); 10 mM NH_4Cl (■); 0.4 mM chloroquine (○); 10 mM methylamine (△) and 0.1 mM dansyl cadaverine (□) before exposure to cholera toxin (10 $\mu\text{g}/\text{ml}$). Samples were taken at various time intervals for assessment of adenylate cyclase activity after breaking the cells.

such that the A subunit is able to penetrate through the bilayer and interact with the guanine nucleotide regulatory subunit of adenylate cyclase at the cytosol side of the membrane. However, besides the obvious thermodynamic difficulties this poses, if the lag period did reflect such an event it is difficult to see why it should be affected by toxin concentration [6] and also why it is apparently unaffected by covalently cross-linking the A and B subunits together [20]. Furthermore, the A chain of cholera toxin does not appear to be inserted into the bilayer when the toxin binds to ganglioside-containing lipid vesicles [21]. Thus the direct penetration of the A subunit through the plasma membrane appears to be unlikely.

Fluorescent-labelled cholera toxin has been demonstrated to bind to cell surface gangliosides, where it undergoes lateral redistribution into patches and in some instances can form caps at one pole of the cell [7,8,19]. This demonstrates that toxin bound to the membrane is mobile in the plane of the membrane.

Many multivalent ligands that aggregate cell-surface receptors are recruited to coated pits whereupon they are rapidly internalised in endocytotic (coated) vesicles (see [10,11,22]). Coated vesicles have been shown to be involved in plasma membrane retrieval, membrane recycling and exocytosis [10,23,24] as well as endocytosis, thus providing a shuttle system between the plasma and intracellular membranes.

Pre-treatment of cells with NH_4Cl , aliphatic amines, chloroquine and dansyl cadaverine has been shown to inhibit the infectivity of various viruses which enter the cell through coated vesicles [25–27]; to inhibit the receptor-mediated endocytosis of α_2 -macroglobulin [11]; to inhibit the entry of the toxic lectin, modeccin into target cells [28]; to inhibit the receptor-mediated uptake of lysosomal enzymes into fibroblasts [29]; to block the action of pseudomonas exotoxin on mouse LM-fibroblasts [30] and to block the action of diphtheria toxin [31,32]. All of these basic compounds are rapidly taken up by cells and concentrated within the lysosomes where they raise the pH of this compartment [13,25] and inhibit the action of lysosomal proteases [13,33,34].

If hepatocytes, prior to their exposure to cholera toxin, are incubated with either of ammonium chloride (10 mM), dansyl cadaverine (100 μM), methylamine (10 mM) or chloroquine (400 μM) then marked effects are seen on both the lag of onset of adenylate cyclase activation and the rate at which activation ensues (fig.1). All of these agents act to markedly extend the length of the lag period and, with the exception of dansyl cadaverine, they decrease the rate at which activation of adenylate cyclase occurs. Cholera toxin acts on adenylate cyclase by virtue of the ability of the A subunit to cause the ribosylation of the guanine nucleotide regulatory protein [3] in a process that requires NAD^+ and GTP. It is unlikely that these agents exert their effects through decreasing the NAD^+ or GTP concentrations as hepatocytes treated with a number of these agents have been shown to be metabolically competent although their rates of protein degradation are significantly decreased [35,36]. Furthermore, we have shown [6] that in hepatocytes the lag period and rate of adenylate cyclase activation by cholera toxin are relatively insensitive to rather larger changes in both the cytoplasmic NAD^+/NADH ratio and the cytoplasmic ATP concentration.

We suggest that the active, A, subunit of cholera

toxin becomes available at the cytoplasmic surface of the plasma membrane by virtue of the toxin being endocytosed, processed in the lysosomes and re-cycled back to the plasma membrane. We envisage that lysosomal processing would lead to the insertion of the A subunit through to the cytosol surface of the endocytosed vesicle membrane. Membranes containing the A subunit would be re-cycled back to the plasma membrane where the toxin could interact with and activate all adenylate cyclase units through the ability of these components to undergo free lateral diffusion in the bilayer [37,38]. Support for our contention comes from observations that cholera toxin has been shown to undergo endocytosis by neuroblastoma cells [39]. Indeed, after endocytosis, cholera toxin can be seen associated with the golgi or GERL complex which have been shown to be involved in the re-cycling of internalised undegraded plasma membrane [39]. The compounds tested may then exert their action on this process at two points. They could either inhibit endocytosis or prevent the insertion of the A subunit into the membrane during processing and re-cycling. There are indeed precedents for both of these suggested modes of action.

- (i) Dansyl cadaverine and methylamine have been demonstrated to block the receptor mediated endocytosis of α_2 -macroglobulin by a mechanism which is thought to involve inhibition of transglutaminase [11].
- (ii) Whilst the various lysomotropic agents we have tested did not inhibit the receptor-mediated endocytosis of intact Semliki Forest Virus into animal cells, they did prevent the spike proteins in the viral envelope from integrating into the membrane of the endocytosed vesicle in order to form a channel for the nucleocapsid to pass across this membrane and enter the cytoplasm [25].

Lysosomal processing is believed to play a key role in the mechanism of entry and action of toxins into cells, as the action of many plant, bacterial and animal toxins, including diphtheria toxin which is structurally related to cholera toxin, is inhibited by lysomotropic agents [28,40]. The mechanism by which these agents achieve their effects is far from clear. However as cross-linked toxin can activate adenylate cyclase [20] it is possible that proteolysis of the endocytosed toxin may be important in allowing the release of the A-subunit and its subsequent penetration across the membrane. Recycling of undegraded plasma membrane from lysosomes has been demon-

strated to be a rapid process in a number of cell types [41,42] and indeed recycling of plasma membrane in hepatocytes occurs at a rapid rate [42]. This, coupled with the ability of relatively small numbers of cholera toxin molecules to activate the entire pool of adenylate cyclase [6] by collisions during free lateral diffusion, could account for the duration of the well-defined lag period before activation is seen in intact cells. Thus cholera toxin may enter and act on cells by a similar mechanism to that used by many other toxins.

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